The Role of DNA Repair Systems in Antibiotic Resistance Acquisition: Using CRISPR-Cas9 to Compare HDR and MMEJ in Escherichia coli K-12

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Abstract

Antibiotic resistance is a major challenge in healthcare. A better understanding of how bacteria can develop antibiotic resistance is needed in order to develop more effective antibiotics. This study investigates how bacterial DNA repair mechanisms might contribute to antibiotic resistance, using *E. coli* K12 and the antibiotic streptomycin as a model system. When *E. coli* experience double-stranded breaks in their DNA, they repair the damage using either homology-directed repair (HDR), which uses a DNA template to repair the break, or microhomology-mediated end joining (MMEJ), which often causes random mutations by using small, non-homologous DNA segments. These two repair mechanisms were examined in the rpsL gene, which encodes a protein in *E. coli*'s 30S ribosomal subunit, the target of streptomycin and other aminoglycoside antibiotics. Using CRISPR-Cas9 technology, targeted double-stranded breaks were induced in the rpsL gene of *E. coli*, and two distinct repair approaches were tested: (a) template-present editing: using HDR to insert a synthetic template containing a K43T resistance-conferring mutation as a positive control, and (b) template-absent editing: forcing MMEJ to create mutations. The bacteria were then grown on streptomycin media to assess resistance development. Template-present editing produced many resistant colonies, confirming the effects of the K43T mutation. More importantly, template-absent modification produced a limited number of resistant colonies, demonstrating that natural MMEJ-induced mutations in the rpsL gene can confer antibiotic resistance. These findings improve our understanding of how DNA repair mechanisms can inadvertently lead to antibiotic resistance and provide crucial insights for developing more effective treatments and monitoring strategies.

Introduction

Antibiotic resistance is currently one of the biggest challenges in modern medicine. Each year, over 2.8 million Americans develop antibiotic-resistant infections, resulting in more than 35,000 deaths (CDC, 2019). This crisis emerges from bacteria's ability to adapt and survive, even in the presence of previously effective antibiotics. There are many ways that bacteria can develop this resistance, many of which are still not fully understood. Bacteria continue to evolve faster than we can develop new antibiotics. Understanding how bacteria develop this resistance is crucial for developing effective strategies to combat it. The purpose of this experiment is to investigate how bacteria can potentially develop resistance through their natural DNA repair mechanisms.

This research uses *E. coli* K-12 and how it develops resistance to streptomycin antibiotics as a model system. Streptomycin belongs to the aminoglycoside family of antibiotics, which are used to treat various gram-negative and gram-positive infections such as urinary tract infections, bacterial meningitis, and tuberculosis. Like other aminoglycosides, streptomycin works by binding to ribosomes (Garneau-Tsodikova et al., 2015). It specifically kills *E. coli* by binding to the 30S ribosomal subunit protein of their ribosome, inhibiting protein synthesis. This ribosomal subunit (specifically the S12 protein of it) is encoded by the rpsL gene. Previous research has shown that a specific lysine to threonine mutation at position 43 (K43T) in the rpsL gene confers resistance by reducing antibiotic binding affinity between streptomycin and the 30S subunit (Pelchovich et al., 2013). However, the K43T mutation is extremely unlikely to occur through *E.*

coli's natural DNA repair mechanisms as it involves a nucleotide substitution (Seol et al., 2018), and there is limited research on how DNA repair-induced mutations in the rpsL gene of *E. coli* could affect streptomycin resistance. Theoretically, if certain mutations occur in a way that alters the binding affinity between streptomycin and the ribosome, it could confer resistance (Masukawa, 1969).

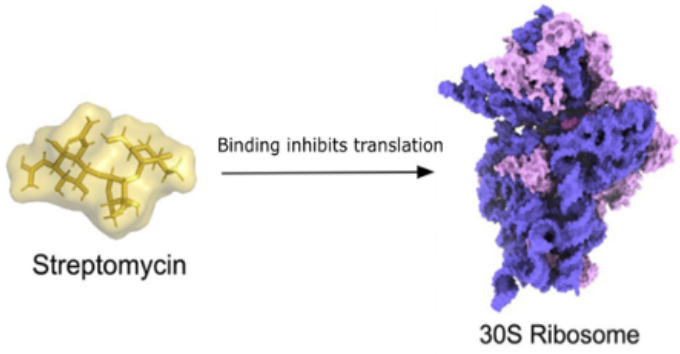


Figure 1: Streptomycin 30s ribosomal subunit interaction (modified from Wei et al., 2022)

In order to understand how random these types of mutations could develop, it's essential to understand how DNA repair mechanisms work. Double-stranded DNA breaks (DSBs) emerge through mistakes in the natural cell process of all bacterial cells or ambient factors such as UV-light and certain chemicals. Bacteria have two different systems, referred to as DNA repair mechanisms, that they can use to repair these breaks: microhomology-mediated end joining (MMEJ) and homology-directed repair (HDR). HDR is a far less error-prone repair system and is unlikely to cause mutations, but it requires a homologous DNA template. The template can be sourced either from the *E. coli*'s sister chromatids or a provided synthetic donor template in laboratory settings and is inserted into the break to repair the DNA (Adar et al., 2009). However, HDR is restricted to certain circumstances and is in competition with MMEJ (Tatiossian et al., 2021). When no homologous template is available, *E. coli* typically resort to MMEJ. This repair pathway uses short homologous sequences (microhomologies) of around 5-25 base pairs at the break sites. During this process, the DNA ends are repaired by inserting these microhomologies, which often results in indels at the repair junction (Sfeir, 2015). Outcomes from MMEJ remain unpredictable, as various mutations have the potential to occur (Martínez-Gálvez, 2024).

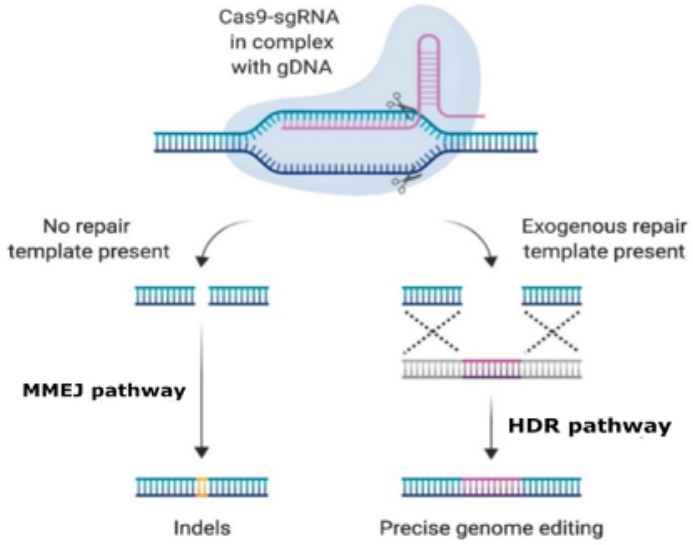


Figure 2: HDR & MMEJ repair of Cas9-mediated DNA breaks (modified from van Kampen, 2019)

Understanding the roles of MMEJ and HDR is essential for this experiment, which aims to compare these repair pathways and their relative effectiveness in generating antibiotic resistance. To trigger and investigate these DNA repair mechanisms, this research utilizes CRISPR-Cas9 technology, a natural prokaryotic defense system that can be programmed to cut DNA at specific locations (Doudna et al., 2014). “Compared to previous gene-editing methods, CRISPR is a lot more efficient, precise, and easy to use” (Zhu, 2022). In this experiment, CRISPR-Cas9 is used to create a DSB in the rpsL gene of *E. coli* K-12. After CRISPR-Cas9 makes the initial cut in the rpsL gene, two different approaches will be used to induce DNA repair: (1) a template-present (TP) approach, which attempts to triggers HDR to insert a synthetic template with a K43T nucleotide change, and (2) a template-absent (TA) approach, which produces the DSB without providing a template, forcing the bacteria to repair the break through MMEJ. A negative control group will also be implemented to validate experimental conditions. Streptomycin resistance will be assessed by quantifying colony forming units (CFUs) of *E. coli* grown on antibiotic plates.

This experiment will investigate if bacteria can naturally develop antibiotic resistance through MMEJ. Additionally, HDR with a synthetic template will serve as a controlled validation of the K43T mutation's effects. If template-absent editing ends up being equally or more effective, it would indicate that *E. coli* has the ability to gain streptomycin resistance not just through targeted modifications, but also from natural MMEJ-induced mutations. Understanding the potential for resistance to emerge through natural DNA repair mechanisms has major implications for medical treatments and monitoring strategies.

Hypothesis

If *E. coli* is modified without a DNA repair template, then it will be less likely to develop streptomycin resistance, because the subsequent MMEJ-mediated repairs are unlikely to produce the specific K43T mutation known to confer resistance

Main Objectives

To use *E. coli*'s susceptibility to streptomycin as a model system for antibiotic resistance and

1. Compare DNA repair mechanisms and their effectiveness in generating resistance
2. Confirm the effects of a K43T mutation in the rpsL gene
3. Determine if naturally occurring MMEJ-induced mutations are able to generate resistance
4. Improve understanding of bacterial adaptation mechanisms

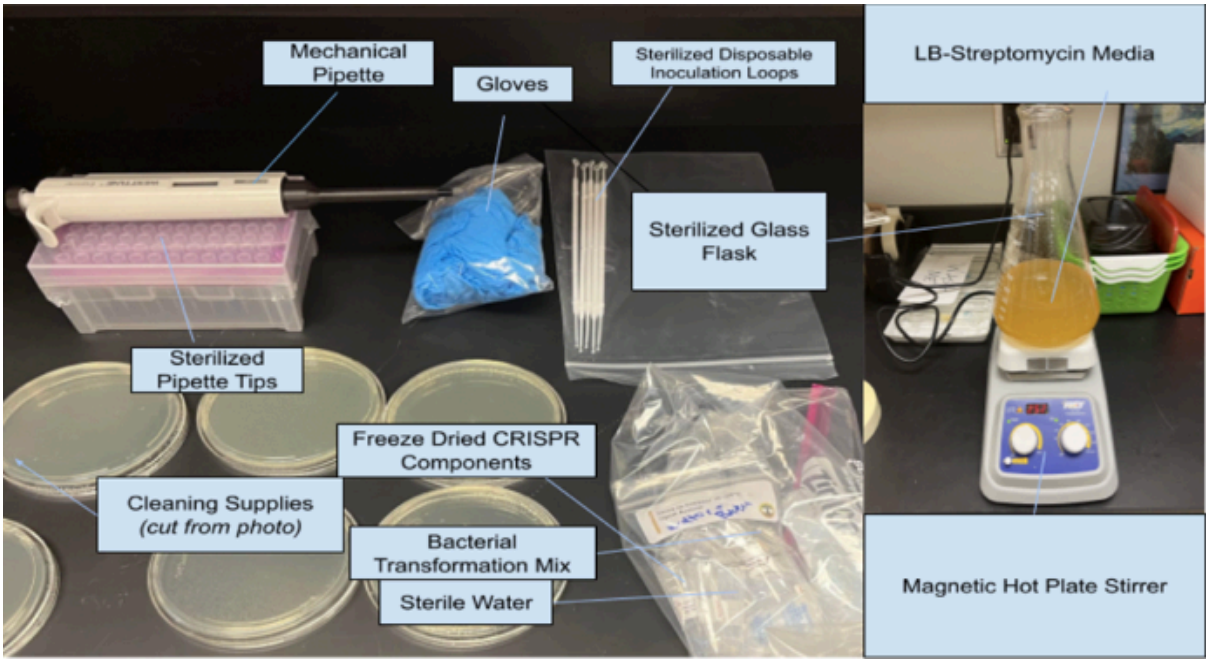


Figure 3: Experimental set-up

Methodology

Preparation of Growth Media: LB-streptomycin plates were prepared by combining 24mL of LB strep media (Streptomycin (50 µg/ml), Kan (25 µg/ml) and Arabinose (1mM)) with 150 mL of tap water in a sterile flask. The solution was heated until homogeneous, cooled for 20 minutes, and distributed among 18 petri plates. Plates solidified for 30 minutes before being stored at 4°C.

Preparation CRISPR Components: Three freeze dried CRISPR components were prepared for transformation: (1) a DNA repair template (1mM, K43T, 80µL, sequence in appendix), (2) a gRNA plasmid (100ng/µL, Ampr, 160µL), and (3) a Cas9 plasmid (100ng/µL, Kanr, 160µL), all stored in separate microcentrifuge tubes. Each freeze-dried component was reconstituted with 55µL sterile water pipetted into each tube.

Preparation of Competent Cells: Next, competent cells were prepared. 100µL of bacterial transformation mix (25 mM CaCl2, 20% PEG 8000) was pipetted into an empty microcentrifuge tube. A 5µL inoculation loop was used to scrape bacteria off a nutrient agar plate culture of *E. coli* K-12/MG1655 obtained from Carolina Biological until the loop was completely full. Then the loop of bacteria was transferred into the microcentrifuge tube with the bacterial transformation mix. The bacteria on the loop were stirred in. Sixteen tubes of competent cell mixtures were prepared in total for the experimental groups (8 replicates per each). Half the tubes were labeled TA (template-absent group), and the other half were labeled TP (template-present group).

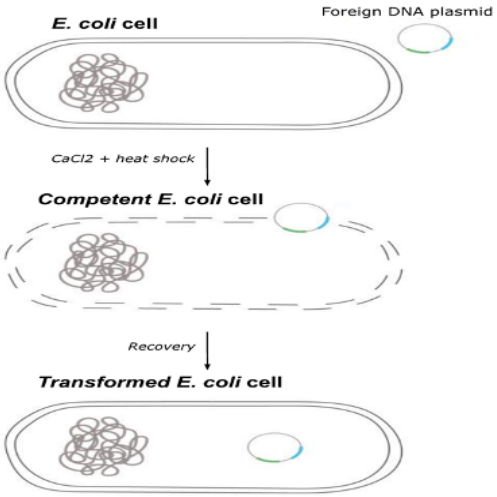


Figure 4: Transformation of *E. coli* cells (designed in Canva)

CRISPR-Cas9 Transformation: The transformation procedure varied between experimental groups as follows:

Template-present Group (8x): Each prepared tube of competent cell mixture received the following via pipetting (pipette tip switched between each addition):
10µL each: Cas9 plasmid solution, gRNA solution, and template DNA solution.

Template-absent Group (8x): The other 8 microcentrifuge tubes of prepared competent cell mixture received the following via pipetting (pipette tip switched between each addition):
10µL each: Cas9 plasmid solution and gRNA solution

All tubes were labeled by group: template-absent (“TA”), and template-present (“TP”). All tubes underwent the following transformation protocol:

1. Cold incubation (4°C, 30 minutes)
2. Heat shock (42°C, 30 seconds)
3. Addition of 250µL recovery media (50µL LB broth/200µL sterile water)
4. Recovery incubation (30°C, 6 hours)

Antibiotic Selection: The entire contents of each transformation tube were pipetted onto individual LB-strep plates and spread with a sterile inoculation loop. For the negative control group, untransformed *E. coli* (from the nutrient agar plate) was transferred directly to two LB-strep plates via sterilized inoculation loops. Plates were labeled according to which group of bacteria was plated onto them and sealed shut with tape. All plates were incubated at room temperature (20°C ±2°C) for 72 hours.

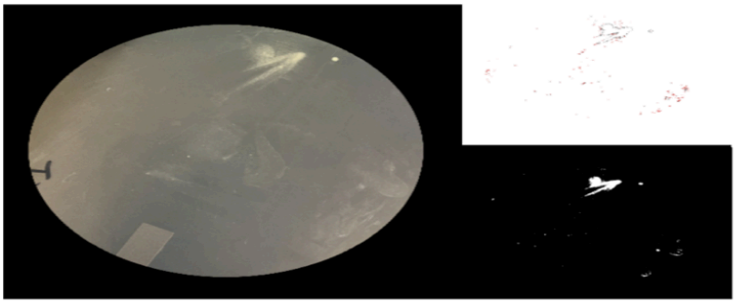
CFU Analysis: Colonies were counted using ImageJ analysis software. Colony counts were all recorded and organized on a google doc.

Laboratory safety protocols: All experimental procedures were conducted in a BSL-1 laboratory under the direct supervision of a qualified biology teacher. Eye protection, protective clothing, and closed-toe shoes were worn at all times and nitrile gloves were changed between procedures to prevent cross-contamination. The bacteria used, *E. coli* K-12, is a non-pathogenic BSL-1 strain specifically designed for educational use. All DNA plasmids were sourced from an approved commercial CRISPR kit, and the use of recombinant DNA in the experimentation was

in accordance with the revised NIH Guidelines for Research Involving Recombinant DNA Molecules and the rules contained in the latest edition of the VJAS Handbook. Work surfaces were disinfected before and after the procedure using a commercial antibacterial spray. Aseptic technique was maintained throughout the experiment: sterile pipette tips were used for all liquid transfers, and plates were properly sealed with tape during incubation. Bacterial plates and disposable materials were disposed of following ISEF and institutional guidelines (see methodology). Lastly, heat protection gloves were worn when using the hotplate.

Results

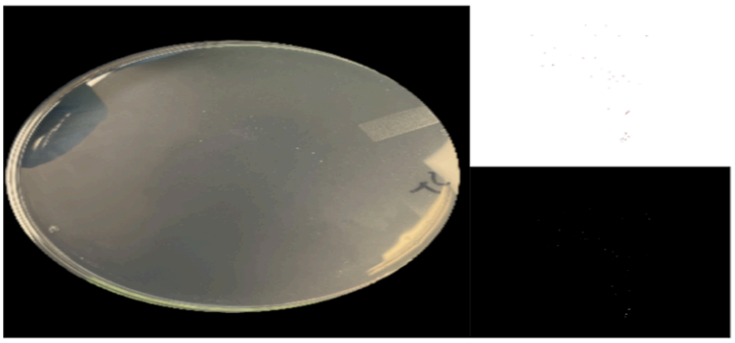
Trial 2 - TP group: bigger more developed colonies of *E. coli* were seen as well as some satellites.



Summary

Slice	Count	Total Area	Average Size	%Area	Mean
temp22.jpg	272	45180	166.103	1.724	151.561

Trial 3 TA group: a few smaller dispersed satellite-colonies can be seen indicating streptomycin was more successful in restricting growth.



Summary

Slice	Count	Total Area	Average Size	%Area	Mean
NOTEMP33.jpg	39	1276	32.718	0.010	255

Figure 5: The growth of small white dots suggests the potential success of the genetic editing experiment. However, it cannot be confirmed with certainty that these colonies are *Escherichia coli* K12/MG1655 without further analysis. The size and appearance of these colonies do resemble images of successful gene editing. For accurate confirmation, additional testing methods such as colony PCR, biochemical assays, or sequencing would be necessary. Despite these uncertainties, for the purposes of this experiment and data analysis, it will be assumed that the colonies are *E. coli*.

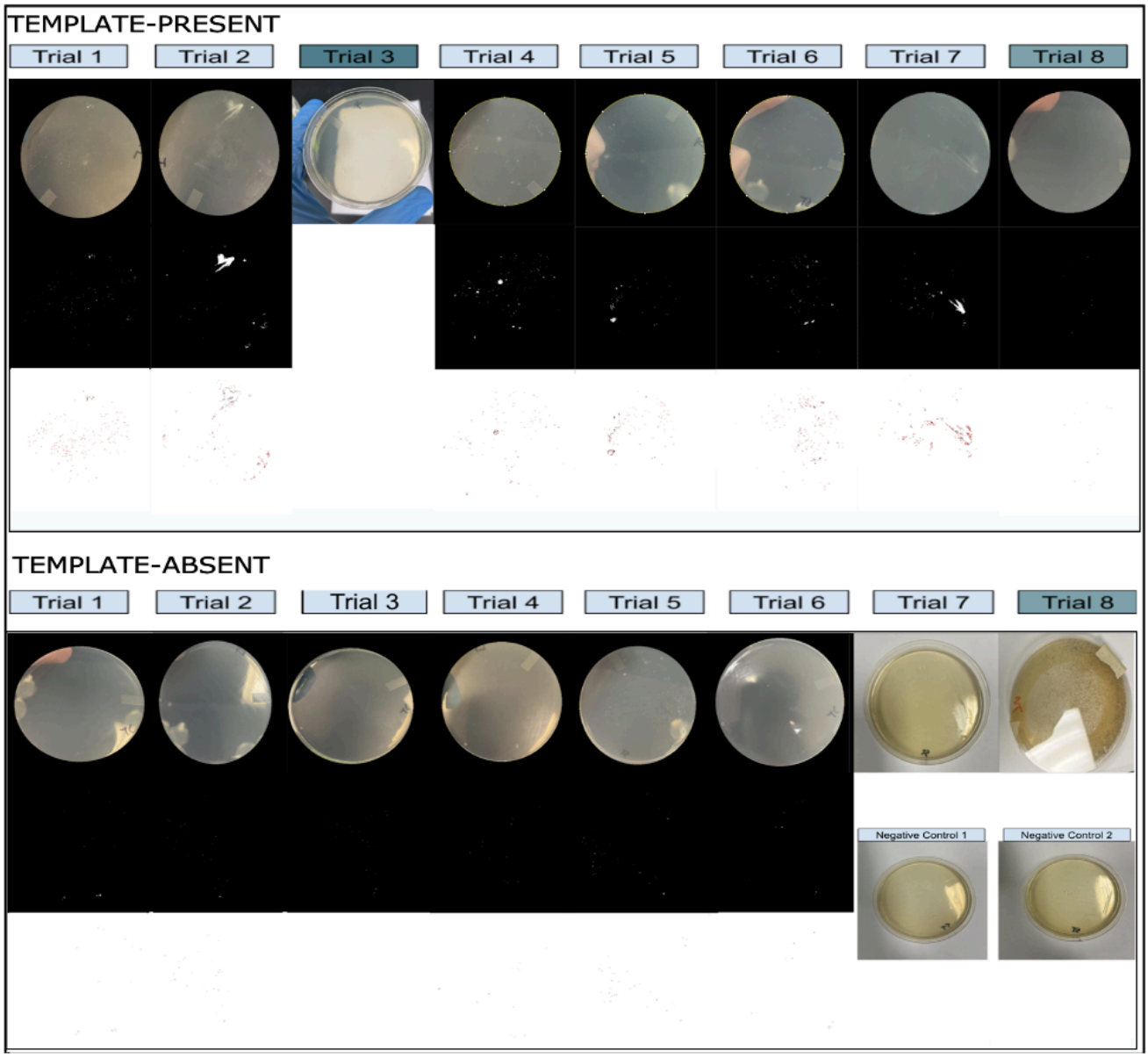


Figure 6: Complete qualitative data table for all trials. Darker blue boxes represent trials with no growth or contamination.

Quantitative Results

Upon initial analysis of the table of results (Figure 7), it was observed that the *E. coli* that underwent template-present CRISPR-Cas9 editing had a higher editing efficacy as this group yielded significantly more CFUs. On the other hand, *E. coli* edited without a DNA repair template showed a maximum yield at only 39 CFUs seen in trial 3. Neither of the two negative control group plates were able to produce any CFUs.

IV – Genetic Modification Approach	DV - Growth of *E. Coli* on Strep Plates After 3 Days of Incubation (CFUs)							
	Trial 1	*Trial 2*	*Trial 3*	*Trial 4*	*Trial 5*	*Trial 6*	*Trial 7*	*Trial 8*
Template-Present	*278*	*272*	*0*	*266*	*172*	*193*	*209*	*17*
Template-Absent	*16*	*33*	*39*	*14*	*34*	*14*	*0*	*0*
No Editing	*0*	*0*	*x*	*x*	*x*	*x*	*x*	*x*

Figure 7: Table of results. Shaded values represent outliers in data.

Trial 3 and trial 8 from the template-present group appeared as outliers as they only yielded 17 and 0 CFUs respectively. These data points significantly inflated the error bars on the initial processed graph due to increasing the standard deviations and additionally lowered the means. Trials 3 and 8 were confirmed as statistical outliers based on the Interquartile Range method. As both values fell below the lower bound of the data set, they were excluded from further analysis.

Additionally, trial 8 from the template-absent group was excluded due to visible contamination (Figure 6). These exclusions were documented in the initial results table and all subsequent statistical analyses were performed without these data points. Means and standard deviations of all the trials in each experimental group were calculated using the Excel 'AVERAGE' and 'STDEV' formula respectively. The means and standard deviations of each group were recorded in a processed data table (Figure 8).

Independent Variable - Genetic Modification Approach	Dependent Variable - *E. Coli* Growth on Streptomycin Media After 3 Days of Incubation		
	Mean ± Uncertainty (CFUs)	Standard Deviation (+/-)	Number of Trials
Template-Present	*232* ± 12	*45.87*	*8*
Template-Absent	*21* ± 1	*14.14*	*8*
No Editing	*0*	*0*	*2*

Figure 8: Processed data table.

The uncertainty in CFU counts was estimated at ±5% of each measurement, accounting for ImageJ software limitations in distinguishing between overlapping colonies, potential variations in image contrast and quality, and the scientifically accepted uncertainty for digital image analysis (De Santo et al., 2004).

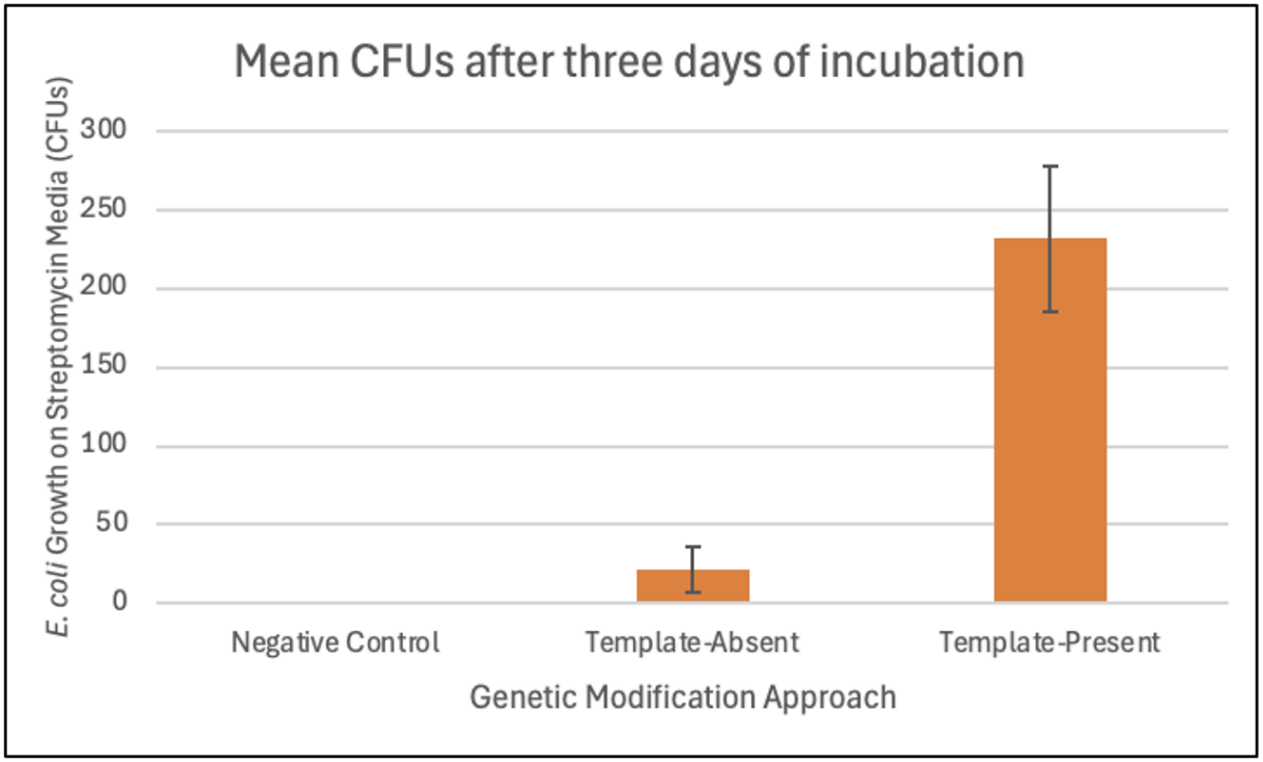


Figure 9: Bar graph of processed data.

A bar graph was used to further visualize the major difference of CFUs yielded between different genetic modification approaches. Both the template-absent and template-present groups showed low to moderate precision of results based on visual analysis of error bars. The large error bars on these groups indicated substantial variability in the standard deviation of CFU counts, which suggested that some variables and other factors remained uncontrolled and inconsistent across trials. Although the error bars were large compared the means of these two groups, they still did not overlap which suggests that the data was statistically significant. To confirm statistical differences between these groups, statistical testing was conducted.

First a two-sample F-test in Excel was used to assess the equality of the variances between the TA and TP groups. Understanding the relationship of the two groups' variances was necessary for selecting the most appropriate type of T-test to conduct. The calculated P(F<=f) one-tail value, or "F-value", was 6.24×10^{-3} which is less than the critical value of 0.05 signifying that the variances between the two groups were not equal and statistically different. Based on the results of this F-Test, an independent samples T-test assuming unequal variance was conducted. This test was appropriate for comparing the two independent experimental groups with unequal variances. The calculated p value of the T-test was 3.73×10^{-5} which was

significantly less than the critical value of 0.05 confirming significant statistical difference between the TA and TP group.

Another F-test was performed to evaluate the equality of variances between the negative control group and TA group. The calculated F-value was undefined because it wasn't possible to compare the variance of the TA group to the variance of the control group, which was 0, as this required dividing a number by zero. However, since the control group had zero variance while the TA group had non-zero variance their variances were inherently unequal. Thus, an independent samples t-test assuming unequal variances was used to determine the statistical significance between the two groups, specifically to better evaluate the efficacy of MMEJ DNA repairs. The p-value from this test was 7.04×10^{-3}, also below the critical value of 0.05, confirming a significant statistical difference between the TA group and control group.

F-test: Two-Sample for Variances			T-test: Two-Sample Assuming Unequal Variances		
Between	F-statistic	Variance Equality	Between	P-value	Significance
TA & TP	6.24×10^{-3}	Unequal	TA & TP	3.73×10^{-5}	YES
TA & Control	Undefined	Unequal	TA & Control	7.04×10^{-3}	YES

Figure 10: Table of statistical tests conducted.

Conclusion

This study investigated how bacteria develop antibiotic resistance through their distinct DNA repair mechanisms by comparing two CRISPR-Cas9 genetic editing approaches in *E. coli*: a synthetic HDR pathway using a synthetic DNA template containing the K43T mutation, and a template-absent MMEJ approach that mimics the natural mutation processes. Streptomycin was used as a model system to represent the various antibiotics in the aminoglycoside class. An unmodified control group was also implemented – the control group's complete inability to grow on streptomycin media validated the experimental conditions. It was predicted that *E. coli* edited with a specific DNA repair template would survive better on streptomycin media. While previous research demonstrated that a specific K43T nucleotide change in the rpsL gene could confer streptomycin resistance, the efficacy of MMEJ in generating antibiotic resistance needed further investigation.

Although constant variables were carefully controlled, it was impossible to maintain complete consistency across trials due to natural experimental conditions. Random errors such as procedural variations and instrumental inconsistencies – e.g., pipette calibration (±1μL), temperature changes during the procedure (±2°C), etc… – likely contributed to larger standard deviations calculated in colony counts but were accounted for in statistical analysis. This experiment was also limited by time and budget constraints. Future research should expand upon these findings by including more replicates and confirming that the bacteria grown on the streptomycin plates were *E. coli* – this could be done through 16S rRNA sequencing or species-specific PCR (Johnson et al., 2019). Furthermore, molecular characterization studies could provide valuable insights by analyzing the rpsL gene sequences from resistant colonies, mapping the specific MMEJ-induced mutations that conferred resistance, and investigating how these mutations correlate with varying levels of antibiotic resistance (different amounts of CFUs).

Statistical analysis revealed significant differences between the results of all three experimental groups, with TP modification yielding approximately 11-fold higher colony formation than the TA approach. This substantial difference not only demonstrated statistical significance ($p < 0.05$) but also indicated a biological impact on editing relating to editing efficiency. The null hypothesis was rejected, and the alternate hypothesis was supported, as TP-induced HDR significantly enhanced bacterial survival in streptomycin-containing environments more than TA-induced MMEJ.

Although the editing efficacy in the TA approach was extremely low, *E. coli* were still able to survive in antibiotic conditions to a limited extent, showing statistically significant difference from the negative control group ($p < 0.05$). This limited survival implies that in some trials appropriate mutations were made that likely (a) altered the 30S protein in a way that prevented streptomycin from binding to it while still remaining functional or (b) affected expression of the rpsL gene in a way that conferred resistance.

Even though the HDR approach with the engineered template produced more resistant colonies, it's important to understand that this pathway was artificially designed to validate the effects of the K43T mutation and does not represent a natural resistance mechanism. In nature, HDR typically repairs DNA precisely without causing mutations. Although the success of TP modification supports the known effects of a K43T mutation in the rpsL gene, the relatively low transformation efficiency suggests that not all *E. coli* cells were able to successfully implement HDR and insert the synthetic template.

More importantly, the limited success of TA modification proved the potential for MMEJ-caused random mutations to generate antibiotic resistance. This finding has many implications for current approaches to combating antibiotic resistance in clinical settings, as it demonstrates how bacteria can develop resistance through their natural DNA repair processes, even without external genetic templates.

Primarily, scientists can use this knowledge to develop more efficient antibiotics. This can include creating drugs that bind to multiple places in the ribosome and maintain effectiveness despite random mutations. These types of antibiotics would combat resistance occurring from MMEJ. Another strategy could be to completely restrict MMEJ in *E. coli.* This could involve using CRISPR to knock out the genes coding for proteins necessary to carry out MMEJ. Additionally, these results can be applied to improve monitoring systems in clinical settings. Medical facilities could create screening protocols that test for both known resistance mutations and for resistance mechanisms that might emerge through MMEJ-like random mutations. This new approach to resistance monitoring would be more efficient and appropriate for identifying new resistance patterns in bacteria.

The significant difference in efficiency between TA and TP editing combined with the viability of both methods demonstrates how complex bacterial DNA repair systems really are. The capability of *E. coli* to develop antibiotic resistance through both precise and random mutations emphasizes the need to start creating more interdisciplinary strategies for combating antibiotic resistance.

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